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Stress response in *Staphylococcus aureus*: regulatory mechanisms influencing enterotoxin gene expression under stress relevant to food production and preservation

PhD Thesis submitted by

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Abstract

Staphylococcus (S.) aureus can give rise to various diseases such as local and systemic infections, as well as toxin-mediated diseases. Staphylococcal food poisoning is caused by staphylococcal enterotoxins preformed in food by enterotoxigenic *S. aureus* strains. In food matrices with other microbial flora, growth of *S. aureus* is inhibited as a result of its poor competitive growth capacity. However, *S. aureus* is relatively resistant to various environmental stresses, exhibiting a competitive growth advantage under conditions of high osmolarity or acid stress.

To date, there is little data specifying the effect of stress on enterotoxin expression encountered under osmotic or acidic conditions. Plasmid encoded classical enterotoxin D (SED) has been suggested to be regulated by the accessory gene regulator (Agr), staphylococcal accessory regulator (SarA), and alternative sigma factor B (σ^B). However, results obtained from previous studies may not be representative since most studies have been conducted using derivatives of *S. aureus* strain NCTC8325 harboring a natural deletion in the *sigB* operon.

The general aim of this thesis was to obtain further knowledge on i) the effect of stress (NaCl, lactic acid, glucose, sodium nitrite) and ii) the effect of regulatory mutations Δagr, $\Delta sarA$, and $\Delta sigB$ on SED expression.

To investigate the effect of stress on *sed* expression, relative *sed* mRNA levels were determined by real-time quantitative PCR under NaCl, lactic acid, glucose, and sodium nitrite stress. In addition, SED protein levels under control and sodium nitrite stress conditions were determined by ELISA. Under NaCl and glucose stress, *sed* expression was generally decreased compared to control conditions. Lactic acid stress (pH = 6.0) did not lead to any significant changes in *sed* expression, while *sed* mRNA levels were increased under sodium nitrite stress. However, SED protein levels were however decreased in the presence of sodium nitrite.

To investigate the effect of regulatory mutations Δagr, $\Delta sarA$, and $\Delta sigB$ on *sed* expression, relative *sed* mRNA levels were determined in wild type (wt) and isogenic regulatory

mutants. The *sed* expression levels of wt and isogenic mutants were compared under control, NaCl, and sodium nitrite stress conditions. Under NaCl stress, *sed* expression was both induced and reduced between wt and Δ*sarA* mutants depending on the strain. In Δ*sigB* mutants, a significant reduction in *sed* expression was observed in one strain. In addition to the mRNA data, the effect of regulatory mutations on extracellular SED protein levels was determined under control and sodium nitrite stress conditions. Both under control and sodium nitrite conditions, SED levels were significantly lower in Δ*sarA* mutants and higher in Δ*sigB* mutants, while no differences were detected between wt and Δ*agr* mutants.

In conclusion, stress data indicates that stressors encountered in food production and preservation influence *sed* expression in a way that cannot be predicted based exclusively on viable cell counts. In addition, data on SED regulation suggests that σ^B and SarA play a role in SED regulation under control and stress conditions, while the importance of Agr in SED regulation may have been overestimated. Because strain-specific differences in response to stress and regulatory mutations occur, inclusion of multiple *S. aureus* strains in future studies is essential. The effect of stress and regulatory mutations should be investigated further in the food matrix both on mRNA and protein level.

Tiivistelmä (Finnish abstract)

Staphylococcus (S.) aureus aiheuttaa monia erilaisia sairauksia kuten paikallisia ja yleistyneitä tulehduksia sekä toksiinivälitteisiä tauteja. Stafylokokkiruokamyrkytyksen aiheuttavat elintarvikkeeseen eritetyt, tiettyjen *S. aureus* -kantojen tuottamat, enterotoksiinit. Muita mikrobeja sisältävissä elintarvikkeissa *S. aureuksen* kasvu on yleensä estynyt, koska se ei pysty kilpailemaan ravinteista muiden mikrobien kanssa. Toisaalta *S. aureus* on suhteellisen vastustuskykyinen useita ympäristön stressitekijöitä kohtaan, mikä antaa sille kilpailuedun tietyissä olosuhteissa kuten korkean osmolariteetin vallitessa tai happamissa olosuhteissa.

Stressitekijöiden kuten osmoottisen stressin ja happostressin vaikutusta enterotoksiinien ilmentymiseen on tutkittu vain osittain. Seuraavien säätelytekijöiden on ehdotettu osallistuvan plasmidin koodaaman enterotoksiini D:n (SED) säätelyyn: Agr (engl. accessory gene regulator), SarA (engl. staphylococcal accessory regulator), ja σ^{B} (vaihtoehtoinen sigma tekijä B). Aikaisempien tutkimusten tulokset eivät kuitenkaan ole välttämättä edustavia, koska tutkimukset on tehty käyttäen *S. aureus* -kannan NCTC8325 johdoksia, jotka kantavat luonnollista mutaatiota *sigB*-operonissa.

Tämän väitöskirjan tavoite oli saada lisätietoa siitä, kuinka i) stressitekijät (NaCl, maitohappo, glukoosi, natriumnitriitti) ja ii) säätelytekijöiden mutaatiot Δagr, $\Delta sarA$, $\Delta sigB$ vaikuttavat SED:n ilmentymiseen.

Stressitekijöiden vaikutuksen tutkimiseksi suhteelliset *sed*-lähetti-RNA:n tasot määritettiin reaaliaikaisella kvantitatiivisella PCR-menetelmällä seuraavien tekijöiden läsnäollessa: NaCl, maitohappo, glukoosi, natriumnitriitti. Lisäksi SED-proteiinitasot määritettiin ELISA-menetelmällä kontrolli- ja natriumnitriittiolosuhteissa. NaCl- ja glukoosistressi aiheuttivat yleisen *sed*-tason laskun verrattuna kontrolliolosuhteisiin. Maitohappostressi (pH = 6.0) ei johtanut tilastollisesti merkittäviin muutoksiin *sed*-tasoissa, kun taas natriumnitriitin läsnäollessa *sed* lähetti-RNA:n tasot olivat korkeammat. SED-proteiinitasot olivat kuitenkin yllättäen alhaisemmat natriumnitriitin läsnäollessa.

Säätelytekijöiden mutaatioiden Δagr, $\Delta sarA$ ja $\Delta sigB$ vaikutuksen tutkimiseksi vil-

lityyppikantojen ja isogeenisten säätelygeenimutanttikantojen *sed*-lähetti-RNA:n tasot määritettiin. Villityypin ja mutanttikantojen sed ilmentymistasoja verrattiin kontrolli-, NaCl- ja natriumnitriittiolosuhteissa. Kontrolliolosuhteissa ei havaittu tilastollisesti merkittäviä muutoksia villityypin ja säätelygeenimutanttien välillä. NaCl:n läsnäollessa Δ*sarA*-mutanttien *sed*-ilmentymistasojen havaittiin joko nousseen tai laskeneen verrattuna villityyppiin, riippuen bakteerikannasta. Tilastollisesti merkittävä lasku *sed*:n ilmentymisessä havaittiin yhdellä Δ*sigB*-mutanttikannalla. Lähetti-RNA-tasojen lisäksi solunulkoinen SED-proteiinitaso määritettiin kontrolli- ja natriumnitriittiolosuhteissa. SED-proteiinitasot olivat molemmissa olosuhteissa alhaisemmat Δ*sarA*-mutanteilla ja korkeammat Δ*sigB*-mutanteilla, kun taas eroa ei todettu olevan villityyppi-kantojen ja Δ*agr*-mutanttien välillä.

Stressitutkimuksista kerätty tieto näyttää osoittavan, että elintarvikkeiden tuotannossa ja säilönnässä käytettävät stressitekijät vaikuttavat *sed*:n ilmentymiseen tavalla, jota ei voida ennustaa ainoastaan elävien solujen lukumäärän perusteella. Lisäksi tutkimustieto säätelygeenien vaikutuksesta antaa viitteitä siitä, että σ^B and SarA ovat tärkeitä SED:n säätelyssä kontrolli- ja stressiolosuhteissa, kun taas Agr:n merkitys voi olla yliarvioitu. Bakteerikantojen stressivasteissa ja säätelytekijöiden toiminnassa havaituista eroista johtuen on välttämätöntä, että tulevaisuudessa tutkimuksiin sisällytetään useita *S. aureus* -kantoja. Stressi- ja säätelytekijöiden vaikutuksen tutkimista tulisi jatkaa elintarvikemalleissa sekä lähetti-RNA- että proteiinitasolla.

List of Thesis Publications

I. Sihto Henna-Maria, Tasara Taurai, Stephan Roger, Johler Sophia, 2014. Validation of reference genes for normalization of qPCR mRNA expression levels in *Staphylococcus aureus* exposed to osmotic and lactic acid stress conditions encountered during food production and preservation. *FEMS Microbiology Letters*, 356:134–140.

II. Sihto Henna-Maria, Tasara Taurai, Stephan Roger, Johler Sophia, 2015. Temporal expression of the staphylococcal enterotoxin D gene under NaCl stress conditions encountered during food production and preservation. *FEMS Microbiology Letters*, 362:1–7.

III. Sihto Henna-Maria, Tasara Taurai, Stephan Roger, Johler Sophia, 2016. Growth behavior and temporal enterotoxin D expression of *Staphylococcus aureus* strains under glucose and lactic acid stress. *Food Control*, 62:69–73.

IV. Sihto Henna-Maria, Budi Susilo Yusak, Tasara Taurai, Rådström Peter, Stephan Roger, Schelin Jenny, Johler Sophia, 2016. Effect of sodium nitrite and regulatory mutations Δagr, $\Delta sarA$, and $\Delta sigB$ on the mRNA and protein levels of staphylococcal enterotoxin D. *Food Control*, 65:37–45.

Additional Publications

V. Johler Sophia, Tichaczek-Dischinger Petra, Rau Jörg, Sihto Henna-Maria, Lehner Angelika, Adam Maja, Stephan Roger, 2013. Outbreak of staphylococcal food poisoning due to SEA-producing *Staphylococcus aureus*. *Foodborne Pathogens and Disease*, 10:1–5.

VI. Ebner Rebecca, Johler Sophia, Sihto Henna-Maria, Stephan Roger, Zweifel Claudio, 2013. Microarray-based characterization of *Staphylococcus aureus* isolates obtained from chicken carcasses. *Journal of Food Protection*, 76:1471–1474.

VII. Rüegsegger Franziska, Corti Sabrina, Sihto Henna-Maria, Johler Sophia, 2014. Toxic bovine mastitis caused by *Staphylococcus aureus* in twin cows. *Schweizer Archiv für Tierheilkunde*, 156:539–542.

Contents

1 Introduction

Foodborne diseases caused by bacteria, viruses, parasites, or toxic substances have occurred since early times. The first written descriptions about food poisoning were made by the ancient Greeks and Romans (Satin 2014). Today, we know significantly more not only about the causes of foodborne diseases, but also about the characteristics of foodborne microorganisms down to the DNA level. The obtained knowledge has essentially contributed to the further development of control methods to improve food safety. Nevertheless, foodborne diseases remain substantial causes of morbidity and mortality worldwide. It has been estimated that annually up to one-third of the population in industrialized countries suffer from foodborne diseases (WHO 2007).

In general, the main factors associated with the increased risk of foodborne outbreaks are i) contaminated raw materials, ii) inadequate cooking, iii) improper storage temperatures, iv) contaminated equipment, and v) poor personal hygiene (**Table 1**) (FDA 2009). These factors are present at all stages of the food processing chain from field to fork. Initially, primary producers are responsible for ensuring that raw materials are safe for consumption or for further processing (risk factors i, iii, iv, v). Actions taken by primary producers or secondary processors of raw materials influence the safety of the food retail products (risk factors i–v). Eventually, restaurants, catering companies, and also consumers have a decisive impact on food safety (risk factors ii–v). The World Health Organization has estimated that about 40% of reported food poisoning outbreaks occur in private homes (WHO 2004). Nevertheless, mismanagement of foodstuffs by food business operators are more likely to affect a larger group of consumers.

Staphylococcal food poisoning (SFP) is an intoxication caused by staphylococcal enterotoxins preformed in food by enterotoxigenic *Staphylococcus (S.) aureus* strains. On average, 240 000 cases of SFP are estimated to occur yearly in the US (Scallan et al. 2011), and 3 000 cases are reported yearly in the EU (EFSA 2015). The real incidence of SFP is considerably higher since most cases are left unreported due to the self-limiting

Table 1. Foodborne illness: Main risk factors and preventive measures.

	Risk factor	Preventive measures
i)	Contaminated raw materials	Good hygiene practices, protective clothing, hygienic design of food manufacturing plant
ii)	Inadequate cooking	Adequate cooking time and temperature
iii)	Improper storage temperatures	No prolonged storage of food at 6–60°C, effective cooling and heating systems
iv)	Contaminated equipment	Separate equipment for handling raw and ready-to-eat foods
v)	Poor personal hygiene	Hand washing, protective clothing, healthy food workers, proper education

nature of the disease. Moreover, not all SFP cases of SFP can reliably be linked to enterotoxins due to insufficient evidence.

The food industry undergoes continuous change as a result of several factors, such as new developments in food processing, introduction of novel foods, and changes in consumer expectations. In recent years, the demand for foods that are less processed and contain fewer preservatives has been increasing. The adaptation of previously validated and widely used preservation methods may, however, introduce unforeseen risks. Moreover, cellular responses to stress related to usage of food preservatives have been only partly investigated (Abee & Wouters 1999; Davidson & Harrison 2002). Within this thesis, we have aimed to gain further insights into the effect of stress on enterotoxin D (SED) expression, as well as regulatory genes influencing SED expression.

This thesis is comprised of four publications referred to in the text by their roman numerals. Thesis objectives are introduced in Chapter 1. Chapters 2–4 give a detailed introduction to *S. aureus*, staphylococcal food poisoning, and enterotoxin regulation. Results of this thesis are discussed in the last two Chapters including future perspectives (5–6). Published or submitted articles are listed at the end of the thesis (**Papers I–IV**).

In **Paper I**, suitable reference genes were validated for normalization of real-time quantitative PCR data under NaCl and lactic acid stress in full and minimal media.

In **Paper II**, the effect of NaCl stress as well as regulatory mutations Δ*agr,* Δ*sarA*, and Δ*sigB* on *sed* transcription was investigated.

In **Paper III**, the effect of glucose and lactic acid stress on *sed* transcription was investigated.

In **Paper IV**, the effect of nitrite stress as well as regulatory mutations Δ*agr,* Δ*sarA*, and Δ*sigB* on *sed* transcription and SED production was investigated.

1.1 Background and thesis objectives

Staphylococcal food poisoning is one of the most prevalent type of foodborne intoxications worldwide. The intoxication is caused by toxins preformed in food by enterotoxigenic *S. aureus* strains. In food matrices with other microbial flora, growth of *S. aureus* is inhibited as a result of the poor competitive growth capacity of *S. aureus*. In contrast, *S. aureus* is relatively resistant to various environmental stresses, exhibiting a competitive advantage under conditions of high osmolarity or acid stress. Currently, food safety criteria set to ensure the microbiological safety of a foodstuff are mostly based on on viable cell counts. However, conditions that may reduce or induce enterotoxin formation are not taken into consideration. While the expression of various enterotoxins under growth conditions without stress has been determined (Akineden et al. 2008; Duquenne et al. 2010; Lee et al. 2007), data is lacking on enterotoxin expression under stress conditions relevant to food production and preservation. Previous studies investigating the effect of stress on SE production have mainly focused on enterotoxin B and rely on immunological methods (Ewald & Notermans 1988; Genigeorgis et al. 1971; Genigeorgis & Sadler 1966; McLean, Lilly & Alford 1968; Troller 1971). They also lack the temporal analysis of enterotoxin production.

The expression of several enterotoxins is controlled by a network of different regulatory elements, whose activity is partially dependent on environmental conditions. The accessory gene regulator (Agr), staphylococcal accessory regulator (SarA), and alternative sigma factor B (σ^B) have been shown to be involved in the regulation of *seb*, *sec*, and

sed expression. Data is limited, however, especially considering enterotoxin regulation under stress conditions. Moreover, most studies investigating the effect of regulatory mutations have been conducted using derivatives of strain NCTC8325 harboring an 11-base deletion in *rsbU*, a gene encoding an indirect positive regulator of σ^B (Gertz et al. 1999). Since a defect in the *sigB* operon has been shown to affect global regulators Agr, Sar, and Rot, results generated using NCTC8325 derivatives may not be representative (Bischoff, Entenza & Giachino 2001; Cassat et al. 2006; Hsieh, Tseng & Stewart 2008; Lauderdale et al. 2009).

The general aim of this thesis was to obtain further knowledge on the effect of stress and regulatory mutations Δ*agr*, Δ*sarA*, and Δ*sigB* on SED expression. The focus of this study was on SED since it is the most common toxin in outbreaks suggested to be influenced by Agr/SarA/σ^B. Stress conditions were chosen so as to reflect realistic stress levels encountered in food production and preservation. Most strains used were well-characterized wild type strains associated with foodborne outbreaks.

Specific aims were:

1. Establishment of a model system for quantification of the expression of staphylococcal enterotoxin D by real-time quantitative PCR (qPCR), including reference gene validation under four different stress conditions (NaCl, nitrite, lactic acid, glucose).

2. Investigation of *sed* expression under stress conditions relevant to food production: NaCl (4.5%), nitrite (150 mg/l), lactic acid (pH 6.0), and glucose (30%).

3. Construction of regulatory knockout mutants lacking the genes which encode the accessory gene regulator (*agr*), staphylococcal accessory regulator (*sarA*), and alternative sigma factor B (*sigB*).

4. Investigation of *sed* expression in regulatory mutants (Δ*agr,* Δ*sarA*, Δ*sigB*) under control and stress conditions.

2 *Staphylococcus aureus*

S. aureus is a gram-positive, catalase-positive, and coagulase-positive coccus. Its genus name consists of the Greek words *staphyle* (bunch of grapes) and *kókkos* (granule) referring to the growth of spherical staphylococci cells in clusters. The species name, derived from the Latin word *aurum* (gold), describes the yellowish appearance of many S. aureus strains. Staphylococci were first observed by Koch and Pasteur in 1878 and 1880. However, the first detailed studies of staphylococci were published by two surgeons, Ogston and Rosenbach. The genus name staphylococci was given by Ogston who discovered grape-like bacteria in post-operative abscesses (Ogston 1881). Rosenbach succeeded in isolating the organism from abscesses and named them *Staphylococcus aureus* based on their yellow appearance (Rosenbach 1884).

2.1 Virulence determinants

Virulence factors influence the onset and the progression of disease by multiple mechanisms. Specific factors are essential at different stages of infection or immune evasion. The pathogenic potential of *S. aureus* is enhanced by its ability to produce a wide array of virulence factors, as well as its high adaptive capacity. In general, *S. aureus* virulence determinants can be categorized into i) antiphagocytic microbial surface-associated factors, ii) cytotoxic exotoxins, and iii) superantigenic exotoxins (Schlievert et al. 2009). However, selected factors from all categories also have the ability to function as immunomodulators, interfering with the host immune response (Foster 2005).

2.1.1 Antiphagocytic factors

Antiphagocytic microbial surface-associated factors include *e.g.* protein A, fibrinogen binding protein, staphylokinase, and clumping factor A that all inhibit phagocytic engulfment (Foster 2005). Protein A has an ability to bind to the Fc region of immonoglob-

ulin G (IgG). Since neutrophils can only bind to the Fc region of IgG, *S. aureus* is not recognized by the immune system and phagocytosis is prevented. Fibrinogen binding protein inactivates the complement system by binding to complement factor C3, leading to the inhibition of opsonization (Lee, Höök, et al. 2004; Lee, Liang, et al. 2004). Staphylokinase is a plasminogen activator, leading to cleavage of complement factor C3b and IgG, resulting in reduced phagocytosis (Rooijakkers et al. 2005). Clumping factor A causes *S. aureus* binding to fibrinogen molecules as well as the adhesion of bacteria to platelets (McDevitt et al. 1994; Siboo et al. 2001).

2.1.2 Cytotoxic exotoxins

Hemolysins (α-, β-, γ-, δ- hemolysin) and leukocidins (*e.g.* Panton-Valentine leukocidin) are examples of cytotoxic exotoxins that can lyse erythrocytes and/or the cells of leukocytic lineage (Schlievert et al. 2009; Yoong & Torres 2013). Cell damage is mediated by formation of pores in the plasma membrane, leading to osmotic dysregulation and cell lysis. Pore-formation on immune cells can also trigger activation of caspase-1 that activates pyroptosis, *i.e.*, inflammatory programmed cell death coupled with the release of pro-inflammatory cytokines, thus promoting *S. aureus* cell survival.

2.1.3 Pyrogenic toxin superantigens

Pyrogenic toxin superantigens affect the immune system of the host in various ways. Superantigens induce massive proliferation of T-lymphocytes as a result of unspecific binding to the major histocompatibility complex class II (MHC-II) and the T-cell receptor molecule (TCR β-chain) (**Figure 1**) (Fraser 1989; Kappler et al. 1989). While a specific binding of conventional antigen typically leads to activation of $< 0.001\%$ of T-cells, an unspecific binding of superantigen may activate up to 20% of T-cells (Langley & Renno 2011). Consequently, large amounts of cytokines and other pro-inflammatory mediators are released, resulting in fever, toxic shock, or even multiple organ failure (McCormick, Yarwood & Schlievert 2001). Several hypotheses have been postulated to explain how bacteria benefit from superantigen production, *e.g.* increased survival and transmission as a result of interfering effects on the immune system (Spaulding et al. 2013).

So far, the following superantigens have been discovered in *S. aureus*: toxic shock syndrome toxin (TSST-1), staphylococcal enterotoxins (SEA–SEE), newly described enterotoxins (SEG–SEI, SER–SET), and staphylococcal enterotoxin-like toxins (SE*l*J–Q, U–Y) (Ono et al. 2015; Ortega et al. 2010; Wilson et al. 2011). In contrast to the antiphagocytic factors and cytotoxic exotoxins, superantigenic exotoxins are only produced by some *S. aureus* strains. Staphylococcal enterotoxins are introduced more closely in Chapter 3.

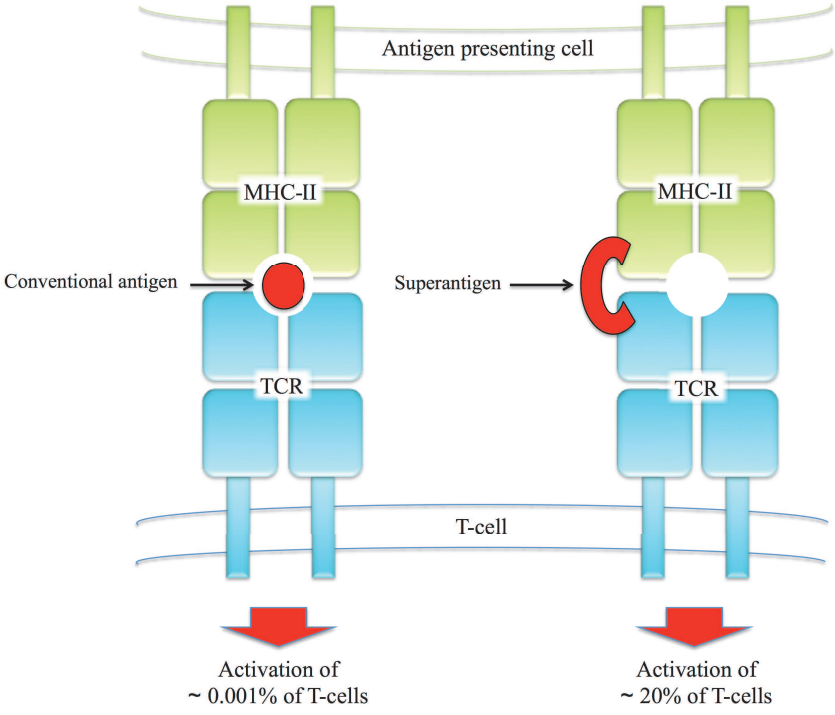

Figure 1. Comparison of conventional antigen and superantigen binding to T-cell receptor (TCR). Conventional antigen bound to the major histocompatibility complex class II molecule (MHC-II) is recognized by T-cell, leading to activation of ~ 0.001% of T-cells. Superantigen binds directly to MHC-II and the T-cell receptor molecule, leading to activation of ~ 20% of T-cells.

2.2 Manifestations of *S. aureus*

As an opportunistic pathogen, *S. aureus* may reside on the skin and mucosal membranes without causing adverse effects on the host, but it also has the capacity to cause a variety of self-limiting to life-threatening infections. Moreover, exotoxins produced by certain *S. aureus* strains can give a rise to toxin-related diseases.

2.2.1 Colonization

S. aureus is a commensal colonizer in humans and animals. In humans, approximately 30% of healthy individuals are permanent carriers of *S. aureus*, and an additional 30% are transient carriers (Halablab et al. 2010; Wertheim et al. 2004). *S. aureus* is found most frequently in the mucous membranes of the nasal cavity, but it is usually also present in other parts of the body, as well (Williams 1963). Colonization rates in different animal species vary: 14–23% in cows (Nagase et al. 2002), 29% in sheep (Vautor et al. 2005), and 90% in chickens (Nagase et al. 2002).

S. aureus colonization is asymptomatic, but the carriers are predisposed to post-surgical infections (Kluytmans, van Belkum & Verbrugh 1995; Levy et al. 2013). In the past, control of nosocomial *S. aureus* infections has focused on preventing cross-infections between patients (Pittet et al. 2000). However, over 80% of the *S. aureus* bacteremia cases that occur after hospitalization have been shown to be of endogenous origin (von Eiff et al. 2001; Wertheim et al. 2004). A local antibiotic treatment with mupirocin has been shown to be an effective method to prevent post-surgical *S. aureus* infections (van Rijen et al. 2008). The effect of decolonization is temporary, however, and resistance to mupirocin in *S. aureus* is a subject of increasing concern (Conly & Johnston 2002; Loeb et al. 2008).

In the case of staphylococcal food poisoning, colonization of the food handler is regarded as an important risk factor since food can be contaminated by the food handler, as a result of unhygienic handling of food (Argudín, Mendoza & Rodicio 2010; Johler et al. 2013; Minor & Marth 1972; Wattinger et al. 2012).

2.2.2 Infections

To establish an infection, *S. aureus* cells adhere to the host cells, multiply, and may also invade host cells and spread systemically through the blood stream (Ribet & Cossart 2015). *S. aureus* causes a wide variety of local and systemic infections in humans and animals. In humans, *S. aureus* is one of the leading causes of bacteremia and infective endocarditis as well as osteoarticular, skin, soft tissue, pleuropulmonary, and device-related infections (Tong et al. 2015). *S aureus* infections have been reported in several animal species such as cattle, sheep, goats, pigs, horses, birds, cats, and dogs (Peton & Le Loir 2014). In dairy animals, *S. aureus* is one of the most common causes of subclinical and clinical mastitis. Since *S. aureus* mastitis treatment is often unsuccessful and recurrent infections are common, control measures focus on prevention of new infections and culling of persistently infected animals (Barkema, Schukken & Zadoks 2006; Quinn et al. 2011).

Continuously evolving resistance mechanisms complicate the treatment of *S. aureus* infections both in humans and animals. Moreover, *S. aureus* does not elicit protective immunity and may reside intracellularly, leading to an increased risk of recurrent infections (Cheung & Otto 2015; Kim et al. 2012; Rogers & Tompsett 1952).

2.2.3 Toxin-related diseases

Some *S. aureus* strains carry one or several toxin genes in their genome, such as genes encoding enterotoxins, toxic-shock syndrome toxin, and exfoliative toxins. Toxin-related illnesses can evolve upon consumption of toxins preformed in food (enterotoxins) or upon exposure to toxins produced in the host under conditions that favor *S. aureus* multiplication (toxic-shock syndrome toxin, exfoliative toxins).

Enterotoxins elicit an emetic response upon ingestion, leading to nausea and vomiting. In addition, enterotoxins function as superantigens that induce a massive proliferation of T-cells and a release of large amounts of pro-inflammatory cytokines. Staphylococcal food poisoning mediated by enterotoxins is discussed more closely in Chapter 3.

Toxic-shock syndrome toxin (TSST-1) belongs to the superantigen family of toxins but does not exhibit emetic activity. TSST-1 causes toxic shock syndrome (TSS) that is primarily associated with the use of high-absorbency tampons, introduced in the late

1970s (Shands et al. 1980). The number of cases has rapidly declined after the connection between tampon usage and TSS was discovered, and preventive measures were taken: these included withdrawal of specific types of tampons and promotion of public awareness about appropriate use of tampons. Non-menstrual TSS can occur in association with different conditions, such as *S. aureus* infections and skin lesions. Influenza-associated TSS may develop as a result of *S. aureus* multiplication in respiratory track epithelium damaged by influenza (MacDonald et al. 1987; McCormick, Yarwood & Schlievert 2001).

Exfoliative toxins (ETs) are responsible for staphylococcal scalded-skin syndrome (SSSS). ETs function as serine proteases that hydrolyze desmosomal proteins between adjacent epithelial cells, leading to the disruption of cell-cell adhesions (Gemmell 1995). Superantigenic activity of ETs is debated, and it has been suggested that superantigenicity of the ETs may not be involved in the pathogenesis of SSSS (Bukowski, Wladyka & Dubin 2010; Ladhani 2001). The localized form of SSSS, also known as bullous impetigo, manifests with fragile fluid-filled blisters on the skin while fever and erythema are present in the generalized form (Ladhani 2001). Untreated SSSS can lead to desquamation of the skin, and in case of infants the loss of the protective epidermis can lead to complications, such as hypothermia, dehydration, and secondary infections. Outbreaks have been reported most commonly in neonatal nurseries (Ladhani et al. 1999).

2.3 Antibiotic resistance

Before antibiotics were discovered, the fatality rate among *S. aureus* bacteremia patients exceeded 80% (Skinner & Keefer 1941). Penicillin, introduced in the early 1940s, improved the prognosis but within a few years, penicillin-resistant staphylococci were discovered. By 1948, over 50% of *S. aureus* strains were found to be resistant to penicillin (Barber & Rozwadowska-Dowzenko 1948). Today, about 80–90% of the human *S. aureus* isolates are resistant to penicillin (Sakoulas & Moellering 2008). The prevalence of penicillin resistance in bovine isolates varies from 5 to 60% in different countries (Erskine et al. 2002; Hendriksen et al. 2008; Kalmus et al. 2011).

Methicillin was introduced in the early 1960s to treat infections caused by penicillin-resistant strains. Emergence of methicillin-resistant *S. aureus* (MRSA) isolates was, however, reported shortly after (Jevons 1961). Initially, MRSA strains were associated with

hospitals (HA-MRSA) and were considered to be restricted to the nosocomial environment. In the early 1990s, MRSA cases that could not be traced to hospitals were reported worldwide and designated as community-associated MRSA (CA-MRSA). The origin of CA-MRSA is not fully elucidated, but since clonal lineages of HA-MRSA and CA-MRSA differ, separate evolutionary origins of HA-MRSA and CA-MRSA have been suggested (Calfee 2011). However, MRSA isolates have also been shown to spread between the community and hospitals (Song et al. 2011). In the mid 2000s, a third type of MRSA now associated with livestock (LA-MRSA) was reported in pigs in the Netherlands and France (Armand-Lefevre et al. 2005; Voss et al. 2005). Subsequently, LA-MRSA strains have been reported in several other countries in Europe as well as in North America and Asia (Huber et al. 2010; Smith & Pearson 2011). Typical LA-MRSA strains belonging to the clonal complex 398 have also been detected in other food-producing animals such as poultry (Nemati et al. 2008), cattle (Monecke et al. 2011), and sheep (Feßler et al. 2012). LA-MRSA has been postulated to have originated when methicillin sensitive *S. aureus* (MSSA) in humans acquired tetracycline and methicillin resistance (Price et al. 2012). Vancomycin has been used to treat MRSA infections, but an increasing number of reports describe the emergence of vancomycin-resistant strains (VRSA) (Tarai, Das & Kumar 2013).

2.4 Growth parameters

S. aureus is a facultative anaerobic, mesophilic bacterium, able to grow at moderate temperatures. Among foodborne pathogens, *S. aureus* is one of the most osmotolerant species being able to grow under conditions with low water activity. In general, *S. aureus* is a poor competitor in the food matrices in the presence of other bacteria. However, in food items with low water activity or low pH, *S. aureus* can exhibit a competitive growth advantage. *S. aureus* growth and enterotoxin formation parameters are summarized in **Table 2**.

Table 2. Selected environmental factors affecting *S.aureus* growth and enterotoxin formation.

Factor	Growth		Enterotoxin formation		References
	Optimal	Min–Max	Optimal	Min–Max	
Temperature	35–41°C	6–48°C	34–40°C	10–48°C	1–8
Water activity	0.99	≥ 0.83	0.99	≥ 0.86	1, 9, 10
NaCl	0%	0–20%	0%	$< 12\%$	1, 11, 12
pH	6–7	4–10	7–8	5–9.6	1, 4, 13, 14

(References: (1) ICMSF 1996; (2) Yang, Yu & Chou 2001; (3) Genigeorgis, Riemann & Sadler 1969; (4) Tatini 1973; (5) Angelotti, Foter & Lewis 1961; (6) Iandolo, Ordal & Witter 1964; (7) McLean, Lilly & Alford 1968; (8) Schmitt, Schuler-Schmidt & Schmidt-Lorenz 1990; (9) Scott 1953; (10) Troller 1976; (11) Genigeorgis et al. 1971; (12) Nunheimer & Fabian 1940; (13) Barber & Deibel 1972; (14) Smith, Buchanan & Palumbo 1983)

2.5 Food preservation and stress response

Food preservation methods aim to inhibit or decrease microbial growth and can be divided into physical and chemical methods (Gustafson & Wilkinson 2005). Physical methods include dehydration, refrigeration and freezing, vacuum and modified atmosphere packaging, heat treatment, UV-irradiation, and pressurization. Different compounds, such as NaCl and organic acids, can be used as preservatives to restrict microbial growth by chemical methods. *S. aureus* exhibits several stress response mechanisms to survive under different environmental conditions. In food production and preservation, the most frequently confronted stress conditions include osmotic and acid stress. *S. aureus* is a highly adaptable organism and even subtle changes in environmental conditions can affect a cell's physiology (Chan & Foster 1998a). Notably, stress resistance of the organism may increase when it is pre-exposed to sublethal stress (Chan et al. 1998). Exposure to one type of stress can also provide cross-protection against other stresses (Cebrián et al. 2010; Pichereau, Hartke & Auffray 2000). In modern food production, the multiple-hurdle principle is commonly applied to control microbial growth, improve food safety, and extend product shelf life. The multiple-hurdle principle is based on the idea that product safety should not depend on a single factor. Moreover, it can provide a strategy on how to reduce the concentrations of single food preservatives required to inhibit microbial growth (Leistner & Grahame 2005).

2.5.1 Osmotic stress

The water activity (a_w) value describes the availability of free water in a specified matrix, such as food. *S. aureus* is one of the most osmotolerant foodborne pathogens as it is able to grow in matrices with $a_w = 0.86$ (some strains can even grow at $a_w = 0.83$) (Scott 1953; Troller 1976). In food products, water activity can be reduced *e.g.* by drying or by adding high concentrations of sugar or salt. The antimicrobial effect of reducing the water activity is based on the microbial requirement to maintain internal turgor pressure. An increase in external osmotic pressure causes water efflux and shrinkage of the cytoplasmic volume what eventually leads to dehydration of a cell (Csonka 1989). Besides regulating the cell volume, bacteria can increase concentration of compatible solutes either by accumulating the solutes from the environment or by biosynthesis (Gustafson & Wilkinson 2005). In comparison to enterococci, *S. aureus* and other staphylococci have been shown to exhibit higher concentrations of compatible solutes and potassium levels, which may partly explain the higher tolerance against osmotic stress (Kunin & Rudy 1991). Osmotic stress induces the expression of several proteins, such as chaperones (Qoronfleh, Streips & Wilkinson 1990), alkyl hydroperoxide reductase C (Armstrong-Buisseret, Cole & Stewart 1995), and pyruvate dehydrogenase (Vilhelmsson & Miller 2002). Changes in gene expression are usually partly associated with a general stress response, while other genes are only affected when cells are exposed to specific additives.

Several *S. aureus* virulence determinants have been shown to be repressed under sucrose and NaCl stress, including α-hemolysin, surface protein A, and TSST-1 (Chan & Foster 1998b). By using immunological methods, enterotoxin production has been shown to decrease under NaCl stress (Genigeorgis & Sadler 1966; Genigeorgis et al. 1971; Ewald & Notermans 1988). SEA production is less affected by low a_w compared to SEB–SED production (Troller 1972). In **Paper II**, we investigated the effect of NaCl stress (0.8 M, 4.5%, $a_w = 0.97$) on *sed* transcription and observed a significant decrease in *sed* expression under NaCl stress in most strains ($n = 4$), while a trend towards increased *sed* expression was observed in one strain. Elevated sugar concentrations have also been shown to decrease enterotoxin A, B, and C production in previous studies (Iandolo & Shafer 1977; Jarvis, Lawrence & Pritchard 1975). In **Paper III**, the effect of a high sugar concentration (1.6 M, 30%, $a_w = 0.96$) on *sed* expression was investigated. In our

study, high glucose stress led to decreased *sed* expression, but a significant decrease was observed only in one strain.

2.5.2 Acid stress

In food preservation, acid stress is used to limit or inhibit bacterial growth by adding an acidulant to the food or by enhancing natural fermentation to promote acidification (Doores 2005). The inhibitory effects of acids depend on the acid used, the concentration, the time of exposure, and the buffering capacity of the food. Weak acids, such as acetic or lactic acid are commonly used in food preservation, since only the undissociated form of acid passes membranes freely (Cherrington et al. 1991). Several mechanisms have been suggested to contribute to antimicrobial activity of acids, such as intracellular accumulation of acid anions, inhibition of essential metabolic reactions, reduction in proton motive force, and denaturation of proteins (Theron & Lues 2010). Gram-positive bacteria exhibit several mechanisms to resist a decrease in intracellular pH and cell damage resulting from acid stress: removal of protons, production of alkali, change of the cell wall composition, production of general shock proteins and chaperones, expression and activation of transcriptional regulators, activation of DNA repair mechanisms, and alteration of metabolic properties (Cotter & Hill 2003). Genes shown to be upregulated in *S. aureus* in response to acid stress include urease subunits A, B, and C (*ureABC*), the *nuoF* gene encoding an NADH dehydrogenase, as well as genes involved in oxidative stress (*katA*, *sodA*) and repair mechanisms (*rexAB*, *polA*) (Bore et al. 2007).

Data on the effect of mild acid stress on enterotoxin expression is limited. On the protein level, enterotoxin production has been shown to be at the highest between pH 6–7 (Genigeorgis & Sadler 1966; Genigeorgis et al. 1971). Expression of prophage encoded *sea* has been shown to be induced in the presence of acetic acid (pH 6.0) as a result of prophage induction (Wallin-Carlquist et al. 2010). In **Paper III**, the effect of lactic acid stress (pH 6.0) on *sed* expression was investigated. While lactic acid stress did not lead to significant changes in *sed* expression, a trend towards increased *sed* expression was observed in all three strains.

2.5.3 Sodium nitrite stress

Sodium nitrite ($NaNO_2$) is a widely used food additive contributing to the preservation, red color, and flavor of cured meat. The red color of the meat is retained when myoglobin and hemoglobin react with nitric oxide resulting from the reduction of nitrite. In *Clostridium botulinum*, sodium nitrite has been shown to inhibit growth by interfering with the formation of iron-sulfur clusters (Duncan & Foster 1968; Pierson & Smoot 1982; Reddy, Lancaster & Cornforth 1983). The desired flavor of cured meat is obtained with relatively low levels of nitrite (50 mg/kg) (Mac Donald, Stanley & Usborne 1980). The mechanisms underlying the bactericidal and bacteriostatic action of nitrite are not thoroughly understood, but inhibition of oxygen uptake, uncoupling of oxidative phosphorylation, and inhibition of metabolic enzymes have been described (Tompkin 2005).

The use of nitrates and nitrites as food additives is regulated by commission regulation (EC) No 1129/2011 (EC 2011). While nitrate and nitrite are not carcinogenic themselves, they may form carcinogenic nitrosamines with dietary amines (Scanlan & Issenberg 1975). Moreover, increased nitrosamine production has been documented in relation different heat treatments, such as frying and baking (Herrmann, Duedahl-Olesen & Granby 2015). In food production, nitrites are only permitted toto be used in a mixture of salt or salt substitute, to prevent the risk of accidental excessive concentrations of nitrite in food products (EC 1995).

The effect of sodium nitrite on enterotoxin formation has been only partially investigated. Sodium nitrite has been shown to inhibit *S. aureus* growth and production of SEA at pH values below 7.0 (Tompkin, Ambrosino & Stozek 1973). Without pH stabilization, *S. aureus* growth or staphylococcal enterotoxin B (SEB) production remains unaffected at nitrite concentrations of up to 200 mg/l, according to McLean (McLean, Lilly & Alford 1968). In a food model study using sausages supplemented with nitrite (c = 154 mg/kg), no SEA or SED formation was detected by ELISA despite *S. aureus* growth to 10^7 cfu/g (Bang, Hanson & Drake 2008). In **Paper IV**, the effect of nitrite (c = 150 mg/kg) on *sed* transcription and SED formation was studied. The transcription of *sed* was increased under sodium nitrite stress, while extracellular SED protein levels were decreased.

3 Staphylococcal food poisoning

Staphylococci were first linked to a foodborne disease in 1884 by Vaughan and Sternberg in a case, in which cheese was contaminated with staphylococci (Hennekinne, De Buyser & Dragacci 2012). In 1914, Barber discovered that milk contaminated with *S. albus* (today known as *S. epidermidis*) caused illness when left unrefrigerated (Barber 1914). The association between staphylococcal food poisoning and toxins produced by staphylococci was first demonstrated by Dack et al. (Dack et al. 1930). With the aid of volunteers Dack et al. could show that the ingestion of a cake contaminated with *S. aureus* resulted in typical symptoms of SFP. Similar results were obtained by the consumption of supernatant from the bacterial culture.

3.1 Symptoms and histopathology

Typically, the symptoms of staphylococcal food poisoning develop within 2–6 hours after ingestion of enterotoxins: the symptoms include nausea, abdominal cramps, vomiting, and diarrhea (Tranter 1990). Low-grade fever, general weakness, and dizziness may also occur. The disease is typically self-limiting, with a duration of 1–3 days. Fatalities are rare and range from 0.03% for the general public, to 4.4% for children and the elderly (Holmberg & Blake 1984).

Most information on histopathological changes in the case of SFP has been obtained from experiments conducted on rhesus monkeys. Inflammatory lesions are mostly observed in the stomach and in the upper part of the small intestine (Kent 1966). The stomach becomes hyperemic, and infiltration of neutrophils can be seen in the lamina propria and the epithelium. In the gastric lumen, a mucopurulent exudate is typically observed (Seo & Bohach 2007). Similar but less severe changes are observed in the small intestine. Extensions of crypts, disruption of brush border, and infiltration of phagocytic cells may be seen in the jejunum. Furthermore, acute lymphadenitis in the mesenteric

lymph nodes may occur in the colon.

3.2 Classification of staphylococcal enterotoxins

Emetic activity is the unique feature of staphylococcal enterotoxins that differentiates them from other superantigens. Staphylococcal enterotoxins are assigned letters in the order of their discovery. According to the international nomenclature committee for staphylococcal superantigens, only toxins with proven emetic activity in primates are to be designated as SE (Lina et al. 2004). Related toxins with no proven emetic activity are designated as staphylococcal enterotoxin-like superantigens (SE*l*). The designation can be subsequently changed into SE if emetic activity is shown later. At the moment, enterotoxins are classified into i) classical enterotoxins (SEA–SEE), ii) newly described enterotoxins with some evidence of emetic activity (SEG–SEI, SER–SET), and iii) SE-like toxins with significantly lower or no emetic activity (SE*l*J–Q, U–Y) (**Table 3**). SEA is the most common toxin related to outbreaks, followed by SED and SEB (Holmberg & Blake 1984; Kérouanton et al. 2007; Wieneke, Roberts & Gilbert 1993).

Genes encoding SE or SE*l* are located in accessory genetic elements including plasmids, prophages, *S. aureus* pathogenic islands (SaPIs), the genomic island *v*SA, or next to the staphylococcal cassette chromosome (SCC) (Argudín et al. 2010).

Enterotoxins are resistant to most conditions that destroy the bacteria that produce them, such as low pH (*e.g.* gastric acid in the stomach), heat treatment (*e.g.* pasteurization), and proteolytic enzymes (*e.g.* pepsin or trypsin in the gastrointestinal tract) (Argudín et al. 2010; Spero & Morlock 1978). With regard to food safety, resistance to heat treatment is an especially important factor since enterotoxins are not destroyed at common temperatures used in food processing. For SEA–SED it has been shown that inactivation of enterotoxins requires heating at 121°C for 3 to 15 min, depending on toxin, concentration, and the matrix type (Fung et al. 1973; Tatini 1976). These results describe the loss of immunological activity and cannot be directly extrapolated to biological and emetic activities of enterotoxins.

Table 3. Gene location and emetic activity of classical, newly described, and enterotoxin-like enterotoxins.

Enterotoxin	Gene location	Emetic activity	References
SEA	Prophage	+	1, 2
SEB	Chromosome (SaPI)	+	3, 4
SEC-1/2/3	Chromosome (SaPI)	+	5, 6, 7
SED	Plasmid	+	8, 9
SEE	Prophage	+	10, 11
SEG	Chromosome (*egc*)	(+)	12
SEH	Chromosome (SCC)	(+)	13, 14
SEI	Chromosome (*egc*)	(+)	12
SE*l*J	Plasmid	nd	15
SE*l*K	Chromosome (SaPI)	(+)	16, 17
SE*l*L	Chromosome (SaPI)	(+)/–	17, 18, 19
SE*l*M	Chromosome (*egc*)	(+)	17, 20
SE*l*N	Chromosome (*egc*)	(+)	17, 20
SE*l*O	Chromosome (*egc*)	(+)	17, 20
SE*l*P	Prophage	(+)	17, 21, 22
SE*l*Q	Chromosome (SaPI)	–	17, 23
SER	Plasmid	(+)	24, 25, 26
SES	Plasmid	(+)	26
SET	Plasmid	(+)	26
SE*l*U/U-2	Chromosome (*egc*)	nd	27, 28
SE*l*V	Chromosome (*egc*)	nd	28
SE*l*X	nd	nd	29
SE*l*Y	nd	nd	30

egc = enterotoxin gene cluster; SaPI = *S. aureus* pathogenic island; SCC = staphylococcal cassette chromosome; + = Emetic activity proven in primate model; (+) = Weak emetic activity proven in primate model; – = Absence of emetic activity proven in primate model; nd = Emetic activity not determined in primate model

(References: (1) Casman 1960;(2) Adesiyun & Tatini 1982; (3) Bergdoll et al. 1959; (4) Kent 1966; (5) Bergdoll et al. 1965; (6) Reiser et al. 1984; (7) Bergdoll 1988; (8) Casman et al. 1967; (9) Chang & Bergdoll 1979; (10) (Bergdoll et al. 1971; (11) Borja et al. 1972; (12) Munson et al. 1998; (13) Ren et al. 1994; (14) Su & Wong 1995; (15) Zhang et al. 1998; (16) Orwin et al. 2001; (17) Omoe et al. 2013; (18) Fitzgerald et al. 2001; (19) Orwin et al. 2003; (20) Jarraud et al. 2001; (21) Kuroda et al. 2001; (22) Omoe et al. 2005; (23) Orwin et al. 2002; (24) Omoe et al. 2003; (25) Omoe et al. 2004; (26) Ono et al. 2008); (27) Letertre et al. 2003; (28) Thomas et al. 2006; (29) Wilson et al. 2011; (30) Ono et al. 2015)

Enterotoxins exhibit a high level of structural homology. SE molecules are ellipsoid in form and contain two unequal domains (A and B). A disulfide loop is located in the end of domain B. Based on amino acid sequence comparison, SEs, SE*l*s, and TSST-1 may be grouped into four phylogenetic groups (Thomas et al. 2007). Classical enterotoxins SEA, SED, and SEE belong to group 1, while SEB and SEC belong to group 2. Newly described and enterotoxin-like toxins are found in groups 1–3, except for the recently described SE*l*X that is categorized to group 4 with TSST-1 (Wilson et al. 2011).

3.3 Emetic and superantigenic activity

Emetic and superantigenic activities of enterotoxins are separate functions, but the studies linking one function exclusively to a distinct part of the SE molecule are controversial. Toxins with low or no emetic activity lack the disulfide loop found at the top of the N-terminal domain of other SEs (Dinges, Orwin & Schlievert 2000). However, the loop is not an absolute requirement for emetic activity (Hovde et al. 1994). In studies conducted with SEA, histidine residue 61 has been found to be important for emesis, but not for superantigenic activity (Hoffman et al. 1996). Moreover, Leu48Gly and Phe44Ser mutant forms of SEA and SEB do not show superantigenic activity but are still able to provoke vomiting (Harris et al. 1993). The binding of SEA–SEC to the T-cell receptor has been shown to occur through the shallow cavity between two protein domains A and B, while the MHC-II binding site is in domain B, in the N-terminal oligonucleotide-oligosaccharide binding fold (Dinges, Orwin & Schlievert 2000).

Ingestion of enterotoxins results in nausea and vomiting but not in measurable enterotoxemia, unless extremely large doses of enterotoxin are consumed. The mechanism by which SEs induce vomiting is unclear. Abdominal viscera including stomach and intestine have been indicated as the sites of emetic action for SEs (Sugiyama & Hayama 1965), but specific receptors have not been identified. It has been suggested that submucosal mast cells are one of the target cells for SEA (Ono et al. 2012). Upon activation, serotonin (5-HT) is released and it binds to the 5-HT_3 receptor expressed on enteric nerves. Subsequent depolarization of enteric nerves stimulates vagal afferent fibers which leads to the activation of the emetic center in the brainstem and the vomiting reflex. In the house musk shrew, SEA-induced emesis was shown to be inhibited by the 5-HT synthe-

sis inhibitor and the 5-HT_3 receptor antagonist (Hu et al. 2007). In addition, type-1 cannabinoid receptor agonists inhibit SEA-induced emesis through a reduction in 5-HT release.

All identified staphylococcal enterotoxins exhibit superantigenic activity, including enterotoxin-like toxins without proven emetic activity in primates. In contrast to conventional antigens that are presented on the cell surface of antigen-presenting cells and recognized by a specific population of T-cells, superantigens can directly bind and cross-link T-cell receptors and MHC-II molecules on antigen-presenting cells. The activation of a large population of T-cells leads to a massive release of pro-inflammatory cytokines, resulting in fever, toxic shock, or even multiple organ failure. The signs of systemic toxicity, such as fever, are not typically reported in SFP, because the amount of enterotoxin ingested is often relatively small and SEs exhibit a lower mucosal penetration capacity compared to TSST-1 (Schlievert et al. 2000).

3.4 Enterotoxin detection methods

It is generally considered that *S. aureus* cell counts of $> 10^5 - 10^6$ /g must be reached in order to result in the formation of detectable amounts of SE. Estimates of the minimal intoxication dose in humans (20–100 ng) are based on epidemiological studies (Asao et al. 2003; Evenson et al. 1988). These limits provide a basis for legislative guidelines and regulations for *S. aureus* counts and enterotoxin detection. However, food safety efforts with regard to *S. aureus* are complicated by several factors: i) enterotoxin production may not be directly related to *S. aureus* counts, ii) some strains may produce much higher amounts of enterotoxins, iii) several enterotoxins may be produced by a single strain, iv) not all enterotoxin types can be detected, v) food matrix components may interfere with enterotoxin detection, and vi) heat treated enterotoxins may have lost their serological activity while remaining biologically active. Currently, enterotoxin detection is based on immunological recognition by specific antibodies. Molecular methods, such as enterotoxin multiplex-PCR, can be used to detect enterotoxins genes. However, information about the presence of enterotoxins in food is missing.

VIDAS (enzyme linked fluorescent assay) and RIDASCREEN (enzyme linked immunosorbent assay) are two enterotoxin screening methods validated by the Community

Reference Laboratory (CRL) of the European Union (**Table 4**). Differentiation between enterotoxin types (SEA–SEE) is possible by using RIDASCREEN but not by using VIDAS. TECRA and TRANSIA are other immunosorbent assay kits available. SET-RPLA is based on reverse passive latex agglutination and it enables differentiation between enterotoxin types (SEA–SED). Immunological methods for SEG, SEH, and SEI detection have been developed but are not yet commercially available (Omoe et al. 2002; Su & Lee Wong 1996).

Table 4. Enterotoxin detection kits commercially available.

Detection kit	Detected SE types	Detection limit (ng/ml)	Provider
RIDASCREEN	SEA–SEE	0.2–0.7	R-Biopharm
VIDAS	SEA–SEE	0.1–1.0	BioMérieux
TECRA	SEA–SEE	≤ 1.0	TECRA International Pty Ltd
TRANSIA	SEA–SEE	0.05–0.2	Raisio Diagnostics
SET-RPLA	SEA–SED	0.75	Oxoid

3.5 Risk assessment and regulations

From the viewpoint of the food industry, food safety is ensured by preventative measures depending on the risks linked to a specific product. These measures rely on the principles of good hygiene practices (GHP) as well as the Hazard Analysis and Critical Control Point (HACCP) system. HACCP was originally established by the Pillsbury company, NASA, and the US army to ensure the safety of food products intended for consumption in space (Bauman 1995). Currently, all food business operators, except the primary producers, are legally obliged to implement HACCP. Good hygiene practices apply to all food business operators, including primary producers (Regulation (EC) No 852/2004) (EC 2004).

Specific microbiological criteria for foodstuffs are defined in commission regulation (EC) No 1441/2007, and they are differentiated into process hygiene criteria and food safety criteria (EC 2007) (**Table 5**). Process hygiene criteria indicate if the production process is performed in a good hygienic manner. These criteria define maximum cell den-

sity levels of coagulase-positive staphylococci permitted in food, and they apply during or at the end of the manufacturing process, depending on the food category. Food safety criteria define the acceptability of a foodstuff in terms of its microbiological safety, and they apply during the shelf life of a foodstuff. These criteria define that SEs must not be detected in 25 g of food in any of the sample units.

Microbiological criteria for *S. aureus* counts and enterotoxin detection in foodstuffs are essential to ensure food safety. However, there are several restrictions to these criteria. Firstly, the number of *S. aureus* cells is not always a good indicator for the presence of enterotoxins since not all *S. aureus* strains are enterotoxigenic or express enterotoxins. In addition, even if *S. aureus* cells were destroyed *e.g.* by the heat treatment, the heat resistant enterotoxins might still be biologically active and could cause food poisoning. Secondly, detection of enterotoxins is complex and standard detection methods are limited to classical enterotoxins (SEA–SEE).

Table 5. Process hygiene and food safety criteria according to commission regulation (EC) No 1441/2007.

Food category	Bacteria/ toxins	Sampling plan		Limits		Analytical method	Stage where criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
Cheeses made from raw milk	CPS	5	2	10^4 cfu/g	10^5 cfu/g	EN/ISO 6888-2	At the time during manufacturing process when the number of staphylococci is expected to be highest	Improvements in production hygiene and selection of raw materials. If values $> 10^5$ cfu/g are detected, the cheese batch has to be tested for staphylococcal enterotoxins.
Cheeses made from milk that has undergone a lower heat treatment than pasteurisation and ripened cheeses made from milk or whey that has undergone pasteurisation or a stronger heat treatment	CPS	5	2	10^2 cfu/g	10^3 cfu/g	EN/ISO 6888-1 or 2		
Unripened soft cheeses (fresh cheeses) made from milk or whey that has undergone pasteurisation or a stronger heat treatment	CPS	5	2	10^1 cfu/g	10^2 cfu/g	EN/ISO 6888-1 or 2	End of the manufacturing process	Improvements in production hygiene. If values $> 10^5$ cfu/g are detected, the cheese batch has to be tested for staphylococcal enterotoxins.
Milk powder and whey powder	CPS	5	2	10^1 cfu/g	10^2 cfu/g	EN/ISO 6888-1 or 2	End of the manufacturing process	
Shelled and shucked products of cooked crustaeans and molluscan shellfish	CPS	5	2	10^2 cfu/g	10^3 cfu/g	EN/ISO 6888-1 or 2	End of the manufacturing process	Improvements in production hygiene.
Cheeses, milk powder and whey powder	SEs	5	0	Not detected in 25 g		European screening method of the CRL for coagulase positive staphylococci	Products placed on the market during their shelf life	Not specified.

CPS = Coagulase positive staphylococci; SEs = Staphylococcal enterotoxins; n = number of units comprising the sample; c = maximum allowable number of sample units giving values between m and M

4 Regulation of staphylococcal enterotoxins

The existence of global gene regulators in *S. aureus* was first suggested in the 1970s, when Yoshikawa et al. discovered that the exposure to a mutagenic agent resulted in simultaneous changes in several phenotypic determinants (Yoshikawa et al. 1974). Additionally, the highest rate of production of most *S. aureus* extracellular proteins was shown to occur during post-exponential growth phase, suggesting gene regulation dependent on growth phase (Abbas-Ali & Coleman 1977). To date, several global gene regulators have been characterized in *S. aureus*, including Agr (Recsei et al. 1986), Sar (Cheung et al. 1992), Sae (Giraudo, Cheung & Nagel 1997), σ^{B} (Deora, Tseng & Misra 1997), Rot (McNamara et al. 2000), ArlRS (Fournier & Hooper 2000), SrrAB (Throup et al. 2001; Yarwood 2001), and MgrA (Luong, Newell & Lee 2003).

Out of the classical enterotoxins, the chromosomally encoded SEB and SEC, as well as the plasmid encoded SED, are regulated by the following elements, amongst others: accessory gene regulator (Agr), staphylococcal accessory regulator (SarA), alternative sigma factor B (σ^{B}), and repressor of toxins (Rot). In contrast, the phage encoded SEA is not regulated by Agr (Tremaine, Brockman & Betley 1993) and transcription of *sea* has been shown to be linked to the lifecycle of the SEA-encoding prophage (Sumby & Waldor 2003; Zeaki et al. 2015). The regulation of the phage encoded SEE has not been investigated but presumably regulation is Agr-independent. While *sea* and *see* expression has been shown to be unaffected by bacterial growth, Agr-regulated *seb*, *sec*, and *sed* exhibit a growth dependent temporal expression pattern (Derzelle et al. 2009; Kusch et al. 2011). It has been claimed that in general, these enterotoxins are produced when a cell density of 10^{5}– 10^{6} cfu/ml is reached. The highest increase in temporal *seb*, *sec*, and *sed* expression is observed during the transition from the late exponential to the stationary growth phase. This induction is less pronounced in *sed* compared to *seb* and *sec* (Derzelle et al. 2009).

Growth dependency of SE expression is linked to the function of different regulatory

elements. During the exponential growth phase and initial phase of infection, cells produce mainly surface proteins (*e.g.* protein A, fibrinogen). SarA is active during the exponential growth phase and reaches the maximal expression at the end of the exponential phase (Manna & Cheung 2001). The Agr-system is being activated during the transition to the stationary phase upon an increasing cell density. The activation leads to repressed transcription of cell wall-associated proteins and increased transcription of toxins and exoenzymes. Activation of the Agr-system leads to down-regulation of Rot, leading to increased toxin expression.

Regulatory elements introduced in this chapter influence the expression of several virulence determinants, but data about their impact on enterotoxin production is still limited. Moreover, the regulatory network controlling virulence factor expression in *S. aureus* is highly intertwined and the final outcome is influenced by parallel activation of several regulators.

4.1 Accessory gene regulator (Agr)

The quorum sensing system Agr enables bacteria to sense environmental signals and to modulate the gene expression in response to the changes in population density, as well as to synchronize the response in the bacterial population (Rutherford & Bassler 2012). The Agr system consists of an auto-inducing peptide (AIP), a peptide precursor (AgrD), an export enzyme (AgrB), and a two-component signal transduction system (AgrC and AgrA) (**Figure 2**) (Novick & Geisinger 2008). The auto-activating circuit is initiated in mid-exponential growth phase, when high concentrations of AgrD peptide are produced intracellularly, processed by AgrB, and secreted in the form of AIP from the cell. Peptide-inducible histidine protein kinase AgrC, situated in the membrane, senses extracellular AIP and undergoes ATP-dependent autophosphorylation. The phosphate is further transferred to response regulator AgrA. Activated AgrA upregulates its own promoter P2 and the adjacent promoter P3. The P2 transcript (RNAII) consists of structural genes of the Agr circuit (*agrBDCA*) and P3 controls the transcription of RNAIII, which functions as the regulatory effector of the Agr-system. Besides acting as a regulatory molecule, RNAIII mRNA also encodes δ-hemolysin that has no regulatory function in the Agr circuit (Janzon & Arvidson 1990).

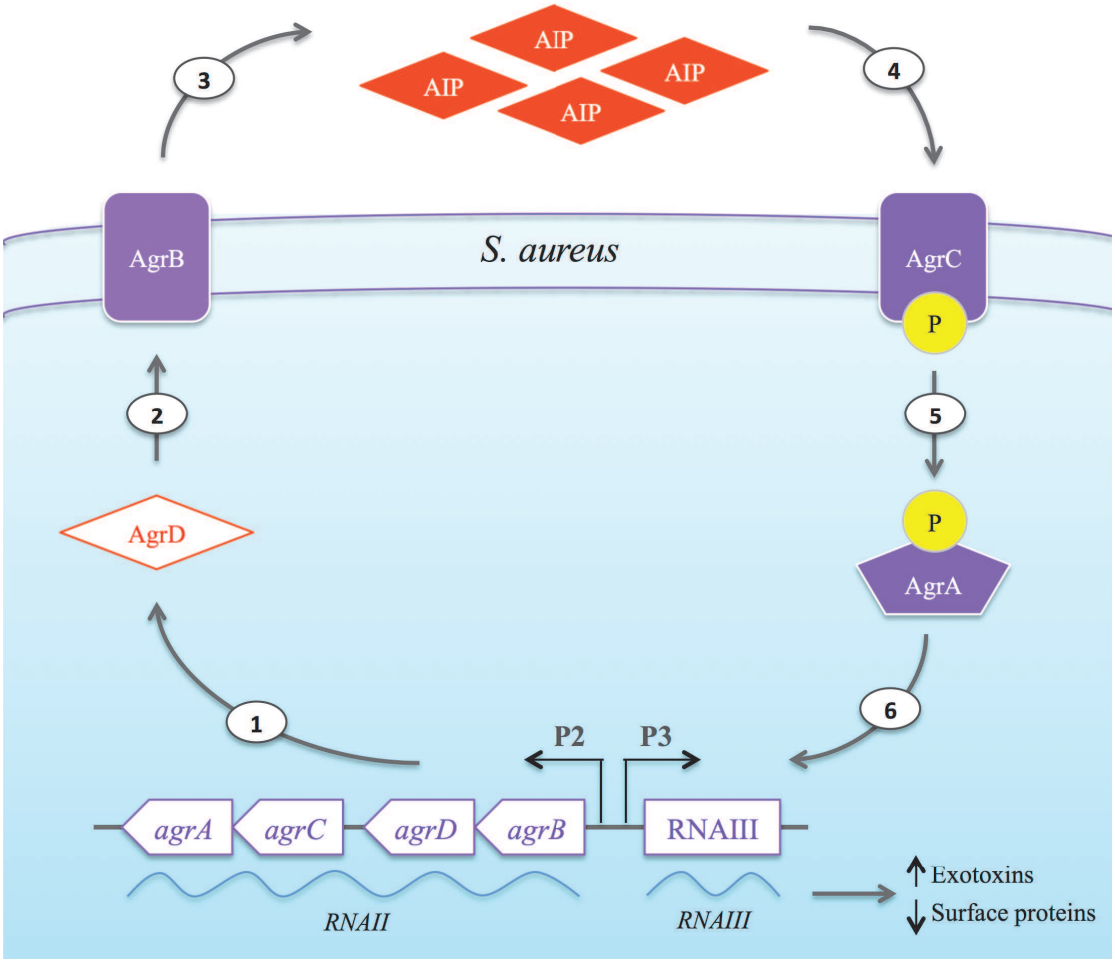

Figure 2. Agr autoactivation circuit.
agrBDCA = accessory gene regulator (BDCA); AIP = autoinducing peptide; P2, P3 = promoter 2 and 3; P = phosphate group

1) Pre-AIP (AgrD) and structural components of the Agr circuit are transcribed as RNAII mRNA.
2) Pre-AIP interacts with the membrane endopeptidase AgrB.
3) Processed pre-AIP is secreted as AIP to the cell exterior.
4) Extracellular AIP binds to and activates receptor domain of AgrC on *S. aureus* surface.
5) Cytoplasmic sensory kinase AgrC is phosporylated and the phosphate is transferred to response regulator AgrA.
6) AgrA activates transcription of two *agr* promoters P2 and P3 leading to increased expression of RNAII and RNAIII. RNAIII functions as regulatory RNA upregulating expression of several virulence factors.

4.1.1 RNAIII

The role of RNAIII in regulatory functions of the Agr-system was first recognized by Novick et al. (Novick et al. 1993). The RNAIII transcript (500 bp) regulates the translation of several virulence factors by multiple mechanisms. RNAIII can either promote or inhibit the translation of the target genes by base pairing with the mRNA of the target gene (Boisset et al. 2007). By this mechanism, translation is promoted if a stem-loop structure of the target gene is relieved upon RNAIII binding, or inhibited if RNAIII masks the ribosomal binding site (Chevalier et al. 2010; Morfeldt et al. 1995). Another post-transcriptional mechanism is mediated through the endoribonuclease RNase III that binds RNAIII in the absence of target mRNA (Lioliou et al. 2012). Binding enables RNAIII to relocate RNase III to different mRNAs, leading to cleavage, destabilization, and reduced translation of the target mRNAs.

Translation of the following secreted virulence factors has been shown to be positively regulated by Agr: toxins (α-, β-, δ-hemolysins), proteases, lipases, enterotoxins, and superantigens (**Table 6**). Surface virulence factors, such as fibronectin or antibody binding proteins, are typically negatively regulated. RNAIII also affects the transcription of several genes, through its inhibitory effect on the transcriptional regulator Rot (discussed in Chapter 4.4). A recent study also suggests that RNAIII stabilizes *mgrA* mRNA, another global transcriptional regulator, leading to increased production of MgrA that affects the expression of several genes (Gupta, Luong & Lee 2015). Some virulence factors, such as phenol-soluble modulins, can be regulated by direct binding of the AgrA reponse regulator, independently of RNAIII (Queck et al. 2008).

In **Papers II** and **IV**, *sed* mRNA levels as well as SED protein levels were compared between wild type strains and their isogenic Δ*agr* mutants. Transcriptional and translational analysis did not reveal any differences between wild type and Δ*agr* mutant strains under control or stress conditions.

Table 6. Selected virulence determinants regulated by Agr, SarA, σ^B, Rot, and Sae.

Protein	Agr	SarA	σ^B	Rot	Sae	References
Toxins						
SEA	0	nd	0	0/–	nd	1, 2, 3, 4,
SEB	+/0	+	–	–	+	2, 7, 6, 9, 8
SEC	+	+	+	nd	nd	9 10, 11
SED	+/0	+	–	–	nd	3, 12, Papers II, IV
SEE	nd	nd	nd	nd	nd	
TSST-1	+	+	+	nd	nd	2, 5, 13
Exfoliatin A, B	+	nd	nd	nd	nd	13, 14
α-hemolysin	+	+	–/0	–	+	13, 15, 16, 17, 18, 19
Surface proteins						
Protein A	–	–	+	–	nd	13, 15, 16, 19
Fibronectin binding protein A–B	–	+	nd	nd	nd	20, 21
Enzymes						
Coagulase	+/–	+	+	+	+	15, 16, 22, 23
Staphylokinase	+	0	nd	nd	nd	13

+ = activation; – = repression; 0 = no effect; nd = not determined

(References: (1) Tremaine, Brockman & Betley 1993; (2) Kusch et al. 2011; (3) Tseng, Zhang & Stewart 2004; (4) Sato'o et al. 2015; (5) Chan & Foster 1998a; (6) Compagnone-Post, Malyankar & Chan 1991; (7) Schmidt et al. 2004; (8) Tseng & Stewart 2005; (9) Regassa, Couch & Betley 1991; (10) Chien et al. 1999; (11) Voyich et al. 2009; (12) Bayles & Iandolo 1989; (13) Recsei et al. 1986; (14) Sheehan et al. 1992; (15) Giraudo, Cheung & Nagel 1997; (16) Saïd-Salim et al. 2003; (17) McNamara et al. 2000; (18) Cheung, Chien & Bayer 1999; (19) Chan et al. 1998; (20) Saravia-Otten, Müller & Arvidson 1997 ; (21) Wolz & Po 2000; (22) Lebeau et al. 1994; (23) Nicholas et al. 1999)

4.2 Staphylococcal accessory regulator (SarA)

SarA is a DNA binding protein that influences the transcription of several virulence factors in a similar fashion to the Agr-system, increasing the expression of exotoxins and decreasing the expression of cell surface proteins (**Table 6**) (Dunman, Murphy & Haney 2001). The *sarA* operon was originally identified after the screening of a Tn917 insertion library for fibrinogen-binding protein-deficient mutants (Cheung et al. 1992). SarA influences gene transcription independently by binding to an A/T-rich recognition motif in the promoter regions of the target genes (Chien et al. 1999). SarA also alters

the gene expression via an *agr*-dependent pathway by activating the *agr* operon, thus increasing RNAII and RNAIII levels (Chien & Cheung 1998). Notably, SarA is required for full expression of *agr* (Blevins et al. 2002; Cheung, Bayer & Heinrichs 1997; Chien, Manna & Cheung 1998).

The *sarA* locus consists of three overlapping transcripts that are transcribed from the three promoters P1, P2, and P3 (Bayer, Heinrichs & Cheung, 1996). P1 and P2 are recognized by the vegetative sigma factor σ^A whereas P3 is dependent on alternative sigma factor σ^B (Deora, Tseng & Misra 1997). P1 and P2 *sarA* transcripts are most abundant during the early exponential growth phase, while the σ^B-dependent P3 transcript dominates during the post-exponential and early stationary phase of growth (Bischoff, Entenza & Giachino 2001; Karlsson & Arvidson 2002).

The effect of loss of SarA was studied in **Papers II** and **IV**. On transcriptional level, no changes were observed under control conditions in *sed* mRNA levels between wild type and Δ*sarA* mutants, while either a significant increase or decrease in *sed* mRNA levels was observed under NaCl stress depending on the strain (**Paper II**). On translational level, SED protein levels were significantly decreased in Δ*sarA* mutants (**Paper IV**).

4.3 Accessory sigma factor B (σ^B)

The RNA polymerase core enzyme is composed of four subunits (α_2, β, β', ω) (Borukhov & Severinov 2002). Binding of the sigma subunit enables RNA polymerase to recognize a promoter and initiate gene transcription. Bacteria typically exhibit several different sigma factors, classified as primary or alternative sigma factors. Primary sigma factors mediate housekeeping gene transcription, while alternative sigma factors aid the transcription of genes needed especially under stress conditions, such as low pH, high osmolarity, energy depletion, and oxidative stress (Kazmierczak, Wiedmann & Boor 2005). In *S. aureus*, three sigma factors have been characterized: one primary sigma factor σ^A (Deora & Misra 1996) and two alternative sigma factors, σ^B(Kullik, Giachino & Fuchs 1998; Wu, de Lencastre & Tomasz 1996) and σ^H (Morikawa et al. 2003).

σ^B is encoded within an operon together with three additional genes *rsbU*, *rsbV*, and *rsbW* (*rsb* = regulator of Sigma B) (Wu, de Lencastre & Tomasz 1996; Palma & Cheung 2001). The activity of σ^B is regulated post-transcriptionally and depends on environmen-

tal conditions (Miyazaki et al. 1999). Under normal conditions, σ^B is bound by RsbW (an antisigma factor) that inhibits the interaction of σ^B with the RNA polymerase core enzyme (**Figure 3**). σ^B can be released from RsbW by the dephosphorylated form of RsbV (an anti-antisigma factor). The dephosphorylation of RsbV is mediated mainly by RsbU but alternative pathways exist, because partial activation of σ^B has been observed in *rsbU* mutants (Palma & Cheung 2001). Stimuli leading to an activation of σ^B–dependent transcription include heat shock, acid or alkaline shock, and hyperosmolarity (Pané-Farré et al. 2006). σ^B activity peaks in the late exponential phase and diminishes towards the stationary phase (Bischoff, Entenza & Giachino 2001).

σ^B has been shown to influence transcription of over 250 genes, including proteins involved in cell wall synthesis, metabolism, and signaling pathways (Bischoff et al. 2004). Among virulence-associated genes, adhesins are upregulated, while transcription of various exoproteins and toxins is repressed (**Table 6**). Notably, σ^B has an opposite effect than Agr on production of several exotoxins (*e.g.* SEB, coagulase, α-hemolysin).

Most studies investigating the effect of regulatory mutations have been conducted using derivatives of strain NCTC8325. This strain harbors an 11-base deletion in *rsbU*, a gene encoding an indirect positive regulator of σ^B as well as a point mutation in *tcaR*, an activator of protein A transcription (Gertz et al. 1999; McCallum et al. 2004). Comparative analysis between NCTC8325 and *rsbU*-repaired NCTC8325 has revealed several differences in phenotypes related to these mutations: differing exoprotein production pattern as well as higher hemolytic activity and lower biofilm formation capacity in NCTC8325 (Beenken, Blevins, & Smeltzer 2003; Herbert et al. 2010). Additionally, expression of other regulatory elements has been shown to be influenced by σ^B. The activity of the *agr* locus has been reported to be higher in both *rsbU* and *rsbUVWsigB* mutants (Bischoff, Entenza & Giachino 2001; Lauderdale et al. 2009). In contrast, increased *sar* activity has not been observed in σ^B deficient mutants (Bischoff, Entenza & Giachino 2001).

The effect of loss of σ^B was investigated in **Papers II** and **IV**. Transcriptional analysis revealed a significant decrease in *sed* mRNA levels between wild type and Δ*sigB* mutant under NaCl stress in one strain (**Paper II**). SED protein levels were, however, significantly increased between wild type and Δ*sigB* mutants in two out of three strains

both under control and nitrite stress conditions (**Paper IV**).

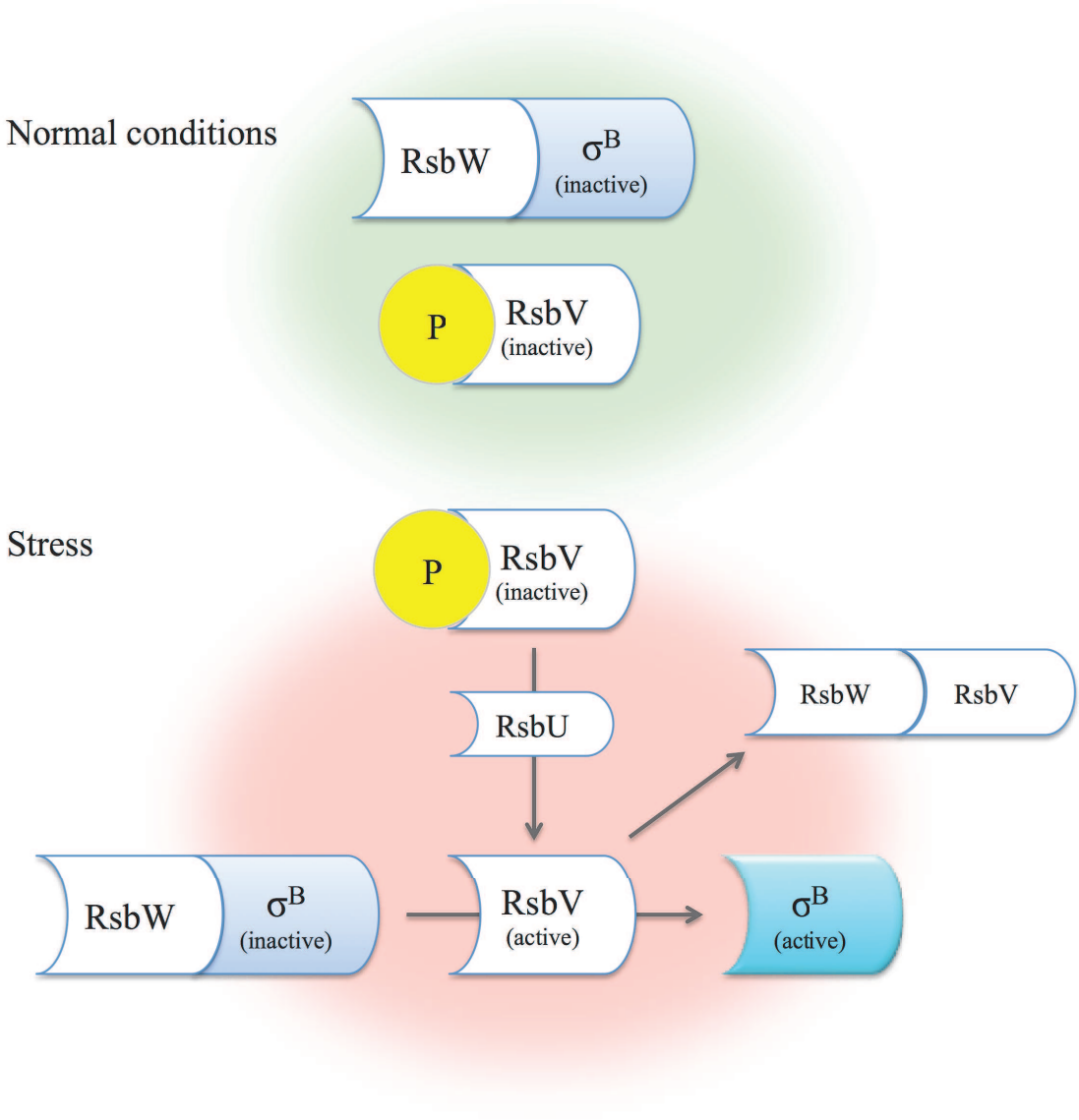

Figure 3. σ^B activation cascade. Under normal conditions, σ^B is bound by RsbW (antisigma factor) that inhibits interaction of σ^B with gene promoter. RsbV (anti-antisigma factor) is phosphorylated (inactive). Under environmental stress, RsbV is dephosphorylated by RsbU, and activated RsbV binds to RsbW, releasing σ^B. Free σ^B binds to the promoter region resulting in upregulation or downregulation of target genes.

4.4 Repressor of toxins (Rot)

Rot is a global transcriptional regulator belonging to the SarA homologues. In general, Rot counters the Agr activity by upregulating translation of surface-associated factors and downregulating translation of secreted proteins (**Table 6**) (Saïd-Salim et al. 2003). Originally Rot was identified when a mutation in *rot* was shown to partially restore the protease and α-toxin activity of an *agr* mutant (McNamara et al. 2000). The mechanism by which Rot regulates transcription or translation of the target genes has remained unclear, but Rot may directly interact with target gene promoters via DNA binding motifs (Killikelly et al. 2015). Out of the classical enterotoxin genes, expression of *sea*, *seb*, and *sed* were shown to be inhibited by *rot* based on changes in promoter activities (Tseng, Zhang & Stewart 2004; Tseng & Stewart 2005). This repressory effect is not common to all enterotoxins, since *rot* was recently shown to stimulate *seh* mRNA expression and SEH production (Sato'o et al. 2015). In the same study, SEA protein levels were shown to be unaffected by Rot, in contrast to the previous study reporting decreased *se*a promoter activity by Rot (Tseng, Zhang & Stewart 2004).

A particularly important interaction between regulatory elements is the inhibition of Rot by RNAIII (Geisinger et al. 2006). Complementary base pairing between RNAIII and rot mRNA sequences leads to a blocking of the ribosomal binding site resulting in inhibition of *rot* mRNA translation (Boisset et al. 2007). RNAIII also promotes cleavage of the *rot* transcript by RNase III (Boisset et al. 2007; Geisinger et al. 2006; Romilly et al. 2012).

4.5 *S. aureus* exoprotein expression (Sae)

Sae is a two-component system involved in global regulation of exoproteins, increasing transcription of α- and β-hemolysins and protein A, amongst others (**Table 6**) (Giraudo, Cheung & Nagel 1997; Giraudo et al. 1999). Data on the involvement of *sae* in enterotoxin regulation is limited. Reduced *seb* and *sec* expression has been reported in *sae* mutants (Kusch et al. 2011; Rogasch et al. 2006; Voyich et al. 2009). Sae has been shown to function downstream of Agr, SarA, and σ^B in the regulatory network, thus having no influence on transcription of these three other regulatory elements (Gi-

raudo, Cheung & Nagel 1997; Novick & Jiang 2003). In contrast, *sae* expression is, to a certain degree, dependent on *agr* (Giraudo et al. 2003) and an indirect effect of *sigB* and *sarA* on *sae* expression has been suggested (Novick & Jiang 2003). Similarly to alternative sigma factor B, *sae* may be activated by environmental stresses such as subinhibitory concentrations of antibiotics, hydrogen peroxide, or α-defensin (Geiger et al. 2008). However, high NaCl and low pH conditions have been shown to decrease *sae* promoter activity (Geiger et al. 2008; Kuroda et al. 2007).

5 Discussion

The aim of this thesis was to gain deeper insights into the effect of stress and the regulatory elements Agr, SarA, and σ^B on enterotoxin D expression. The results are discussed in the three subchapters, including reference gene validation for real-time quantitative PCR (qPCR), the effect of stress on SED expression, and the effect of regulatory mutations on SED expression.

5.1 Reference gene validation for real-time quantitative PCR (Papers I, III, IV)

The objective was to identify reference genes that can be used as endogenous controls for normalization of qPCR mRNA expression levels in food-related stress conditions. Previous studies define the suggested reference genes to be used under control conditions, but reference gene validation has not been conducted under stress conditions, such as were used in this project. Without appropriate reference gene validation, detection of small differences in gene expression is unfeasible and obtained results may even be erroneous (Dheda et al. 2005).

The expression stability of nine reference gene candidates was examined under control conditions and four different stress conditions: NaCl (4.5%), nitrite (150 mg/l), lactic acid (pH 6.0), and glucose (30%). The best-suited reference genes, as well as the recommended number of them, were identified for each condition using BestKeeper and geNorm programs. Under glucose and sodium nitrite stress, housekeeping gene expression was more affected than under NaCl and lactic acid stress. Therefore, the use of three reference genes is recommended for normalization under glucose (**Paper III**) and sodium nitrite stress (**Paper IV**), while two genes may be sufficient under NaCl and lactic acid stress (**Paper I**). It has been previously demonstrated that multiple genes are required for normalization to obtain reliable expression data (Vandesompele et al. 2002).

The *16S rRNA* gene, commonly used in qPCR data normalization in *S. aureus* (Eleaume & Jabbouri 2004; Lee et al. 2007; Stutz, Stephan & Tasara 2011), was not among the best suitable reference gene candidates under the tested conditions. In some previous studies, the use of *16S rRNA* as a single reference gene has also been discouraged, since *16S rRNA* transcripts were shown to exceed most other transcripts in stability (McKillip, Jaykus & Drak 1998) and do not reflect overall mRNA in *S. aureus* (Theis, Skurray & Brown 2007).

The reference gene validation performed may facilitate the selection of reference genes for stress response models of other researchers. However, the results should be regarded as guidelines since the suitability of reference genes should be checked separately for each experimental set-up (Hruz et al. 2011). In our approach, the stability of housekeeping gene expression was evaluated at a single time point and bacterial growth phase. Housekeeping gene expression is, however, often affected not only by the changes in environmental conditions but also depending on the growth phase (Bujold & MacInnes 2015). If relative expression of a target gene will be quantified at several phases of bacterial growth, the expression stability of reference genes should be ideally assessed for multiple growth phases.

5.2 The effect of stress on staphylococcal enterotoxin D gene expression (Papers II – IV)

The majority of previous studies investigating the effect of stress on enterotoxin production were conducted in the 1960s and 1970s, relying exclusively on immunological methods. However, it has been demonstrated that loss of serological recognition does not equal to loss of biological activity (Bennett 2005). Therefore, other methods, such as real-time qPCR, may be beneficial to gain further knowledge on enterotoxin expression under stress conditions. qPCR technology enables rapid, sensitive, and specific relative quantification of enterotoxin expression. Relative gene expression data provides valuable information about changes in temporal enterotoxin expression in response to environmental stresses.

Relative expression of staphylococcal enterotoxin D was defined at different growth phases under control conditions as well as under NaCl, sodium nitrite, lactic acid, and

glucose stress. Under NaCl stress, *sed* expression was significantly decreased in most strains, while a trend towards increased *sed* expression was observed in one strain (**Table 7**) (**Paper II**). Under lactic acid stress, *sed* expression was not significantly altered. However, a trend towards increased *sed* expression was observed in later growth phases (**Paper III**). Glucose stress led to decreased *sed* expression in late stationary phase (**Paper III**).

Table 7. The effect of NaCl, glucose, lactic acid, and nitrite stress on *sed* expression.

Condition	Growth phase			
	Early exponential	Mid-exponential	Early stationary	Late stationary
NaCl (4.5%)	↘↘↗↗↗	↘↘↘↘↘	⇓↘↘↗↗	⇓⇓⇓⇓↗
Glucose (30%)	↘↘↘	↗↘↘	↗↗↗	⇓↘↘
Lactic acid (pH 6.0)	↗↗↘	↗↗↗	↗↗↗	↗↗↗
Nitrite (150 mg/l)	↗↘↘	↗↗↗	↗↗↗	⇑⇑↗

Each arrow represents one strain. ⇑⇓: statistically significant change;
↗↘: non-significant trend.

Regarding the risk related to enterotoxin expression under stress, these results indicate no increased risk associated with high glucose concentrations. In contrast, although the addition of salt generally led to reduction of *sed* expression, salt induced expression may occur in some *S. aureus* strains. Furthermore, the observed trend towards increased *sed* expression under lactic acid stress should be interpreted carefully. Since many *S. aureus* strains carry genes coding for several enterotoxins, food poisoning may occur due to the combined intake of low doses of several enterotoxins. Recently, expression of phage encoded *sea* has been shown to be induced at pH 5.5–6.0 adjusted with acetic acid (Wallin-Carlquist et al. 2010). This kind of stress-induced expression of several enterotoxins could lead to an underestimation of the intoxication risk related to certain foods.

Interestingly, *sed* expression was significantly induced under sodium nitrite stress in two strains, and a clear trend towards induced *sed* expression was observed in the third strain (**Paper IV**). To see if induction could be also detected on protein level, SED ELISA was performed. In general, SED levels were reduced under sodium nitrite stress.

However, in two Δ*agr* regulatory mutants, a trend towards increased SED production in the presence of sodium nitrite was observed. These observations raised two questions: i) why do *sed* mRNA levels not directly indicate SED protein levels and ii) why are SED protein levels increased under sodium nitrite stress in Δ*agr* mutants.

Recent studies investigating *sea* and *sec* have shown that enterotoxin transcription levels do not always reflect extracellular protein levels, particularly under certain environmental stresses (Valihrach et al. 2014; Zeaki et al. 2014; Zeaki, Rådström & Schelin 2015). In *Bacillus cereus*, strain-specific post-transcriptional and post-translational toxin modification has been suggested, affecting mRNA stability, translation initiation, protein durability, and resistance to extracellular degradation (Jeßberger et al. 2015). In our study, relative enterotoxin gene expression levels under control conditions agreed with changes in protein levels defined by ELISA (**Paper IV**, Figures 1 and 2), showing highest *sed* expression and SED production in strain RKI2, followed by RKI1 and SAI48. Lack of correlation between relative expression and protein levels under nitrite stress conditions could be partly attributed to suboptimal environmental conditions. In response to osmotic stress, bacteria accumulate compatible solutes such as proline and glycine betaine to maintain hydrostatic pressure (Miller, Zelt & Bae 1991). It has been hypothesized that this could also cause impaired secretion of exotoxins, leading to reduced amounts of enterotoxin that can be detected extracellularly. Differences in transcriptional and protein levels may also be due to enterotoxin regulation at translational level. Since Agr has been suggested to positively affect SED production mainly on post-transcriptional level, this could offer one explanation for a trend towards induced expression under sodium nitrite stress in Δ*agr* regulatory mutants.

In conclusion, *sed* expression may be either increased or decreased depending on environmental stress, and the influence of stress can also vary depending on the strain. Comparison of mRNA and protein quantification data revealed that mRNA levels do not always reflect extracellular enterotoxin levels under stress conditions. ELISA results generated using polyclonal SED antibodies should be further confirmed using monoclonal SED antibodies, since cross reactivity of polyclonal antibodies with other enterotoxins may introduce bias that is difficult to predict. In general, transcriptional data can still provide essential information about the cellular stress response related to specific

stressors. qPCR is also a powerful tool in detecting enterotoxin mRNA produced in low concentrations. It can also be useful to study gene expression if respective protein detection assays are not yet available.

5.3 The effect of regulatory mutations on staphylococcal enterotoxin D gene expression (Papers II, IV)

Expression of *S. aureus* virulence genes is regulated by several regulatory elements, including Agr, SarA, and σ^B. The impact of knockout mutations on the expression of several virulence genes has been previously investigated using both in vitro and in vivo models. However, most studies have been conducted using *S. aureus* strain NTCT8325 harboring a deletion in the *sigB* operon (Gertz et al. 1999) that in turn affects the function of other regulatory elements such as Agr, Sar, and Rot (Bischoff, Entenza & Giachino 2001; Cassat et al. 2006; Hsieh, Tseng & Stewart 2008; Lauderdale et al. 2009). We aimed to gain more knowledge on the significance of regulatory elements Agr, SarA, and σ^B on enterotoxin D expression in vitro.

The effect of regulatory mutations Δ*agr*, Δ*sarA*, and Δ*sigB* on enterotoxin D expression was investigated under control conditions as well as under NaCl and sodium nitrite stress. Relative *sed* expression was not significantly affected under control conditions between wild type and isogenic regulatory mutants defined by qPCR (**Paper II**). Under 4.5% NaCl stress, *sed* expression was either significantly decreased or increased in Δ*sarA* mutants depending on the strain. In Δ*sigB* mutants, a significant decrease in *sed* expression was observed in one strain (**Paper II**).

Protein levels defined by ELISA showed no significant difference in SED levels in Δ*agr* mutants compared to SED levels in the wild type at the same time point and growth condition (**Paper IV**). In contrast, a significant decrease in SED levels was observed in Δ*sarA* mutants and a significant increase in SED levels in Δ*sigB* mutants compared to the wild type (**Table 8**). Under 150 mg/l nitrite stress, SED protein levels were decreased compared to control conditions in Δ*sarA* and Δ*sigB* mutants, similarly to wild type strains, while a trend towards increased SED production under sodium nitrite stress was observed in most Δ*agr* strains. The effect of regulatory mutations on SED expression was partially strain-specific, based on both mRNA and protein data. It was

hypothesized that this may be due to sequence variation, but sequencing of *sed* genes and *sed* promoter regions of the examined strains did not reveal any sequence variation related to differences in SED regulation.

Table 8. The effect of regulatory mutations on SED protein levels under control (LB)and nitrite stress conditions (Nit) between wild type and isogenic regulatory mutants at the same time point and growth condition.

Time after inoculation (h)	Δ*agr*		Δ*sarA*		Δ*sigB*	
	LB	Nit	LB	Nit	LB	Nit
8	↘↗↗	↗↗↘	↘⇓↗	↘↘↘	↘↗↗	↗↗↘
10	↘↘↗	↗↗↘	↘↘↗	↘↘↘	↘↗↗	↗↗↘
12	↘↘↗	↗↗↘	↘↘↗	↘↗↘	↗↗↘	↗↗↘
24	↗↗↗	↗↗↗	↘↘↗	↘↘↘	⇑⇑↗	↗↗↗

Each arrow represents one strain. ⇑⇓: statistically significant change;
↗↘: non-significant trend.

Agr remained as the single regulatory element whose absence did not lead to any significant changes in *sed* expression or SED production. This may be unexpected since Agr is the most studied regulatory element in *S. aureus* and has been regarded as one of the main positive regulators of several enterotoxins. However, more recent studies indicate that the importance of Agr may have been overestimated as a result of the use of σ^B deficient derivatives of strain NCTC8325. The lack of σ^B activity appears to result in increased RNAIII expression and subsequent overactivation of the Agr-system (Lauderdale et al. 2009). Studies showing decreased production of SEB, SEC, and SED in *agr* mutants (Bayles & Iandolo 1989; Gaskill & Khan 1988; Regassa, Couch & Betley 1991) have been using a strain designated as ISP546 (Mallonee, Glatz & Pattee 1982), belonging to derivatives of NCTC8325. Moreover, Schmidt et al. suggested that Agr is an inducer of *seb* only if the *sigB* operon is not functional (Schmidt et al. 2004). The post-exponential increase in *sed* transcription has also been reported to result from reduction of Rot activity by the Agr-system rather than as a direct effect of Agr (Tseng, Zhang & Stewart 2004).

Data on the effect of the loss of regulatory elements Agr, SarA, and σ^Bsuggests that σ^B and SarA play a role in SED regulation under control and stress conditions, while the importance of Agr in SED regulation may have been overestimated. Moreover, strain-

specific differences in SED regulation were notable. Complementation of mutant strains would be necessary to confirm that the observed changes in phenotype resulted from the loss of the specific regulatory element. Since the regulatory pathways are interconnected, sequencing of regulatory elements would be necessary to identify the factors accounting for strain specific differences in SED regulation.

6 Conclusions and future perspectives

Based on results obtained within this thesis, the following conclusions were drawn about the effect of stress and regulatory mutations on enterotoxin D expression:

- NaCl stress (4.5%) generally leads to decreased *sed* expression. However, in some *S. aureus* strains *sed* expression remains unaltered or may be increased in the presence of NaCl despite the growth retardation. (**Paper II**)

- Glucose stress (30%) decreases *sed* expression, albeit not as pronouncedly as NaCl stress. (**Paper III**)

- Lactic acid stress (pH 6.0) has no negative impact on *sed* expression. Higher *sed* expression levels under lactic acid stress may be encountered despite the adverse effects of stress on growth. (**Paper III**)

- Nitrite stress (150 mg/l) increases *sed* expression. Extracellular SED protein levels are, however, decreased under nitrite stress. (**Paper IV**)

- Even mild stress levels generate considerable changes in housekeeping gene expression. Therefore, reference gene candidates for qPCR should be validated for each study and a multiple reference gene approach for normalization is recommended. (**Papers I, III, IV**)

- σ^B is an important regulator of SED expression both under control and stress conditions. SarA also influences SED formation while the role of Agr in *sed* regulation may have been overestimated in previous studies. SED regulation appears to be influenced by strain-specific differences. (**Papers II, IV**)

In the future, several aspects should be considered to improve knowledge on the characteristics of *S. aureus* related to food safety.

- Since *S. aureus* is a highly versatile and adaptive pathogen, multiple strains originating from different sources should be included in studies investigating the effect of stress and regulatory elements.

- Studies about regulatory elements conducted using σ^B deficient derivatives of strain NCTC8325 should be treated with caution. Consequently, sequencing of functionally interconnected regulatory genes would improve reliability of studies on regulatory genes.

- Determination of both mRNA and protein levels is recommended when investigating the effect of stressors or loss of regulatory genes on enterotoxin levels.

- Possible explanations for the discrepancies observed between the mRNA and protein quantification results under stress conditions should be further examined, *e.g.* by corroborating ELISA results using monoclonal antibodies and by investigating the role of post-transcriptional regulation and toxin secretion under stress conditions.

- Potential stress-induced *sed* expression related to lactic acid and NaCl stress should be investigated on protein level.

- Additional food matrix experiments are a prerequisite to draw final conclusions about the effect of stress or regulators in specific food environments.

- Enterotoxin detection methods should be further developed to enable detection of low quantities of enterotoxins and the detection of newly described enterotoxins.

- Legislative guidelines defining the limits for staphylococcal counts in different foodstuffs need to be regularly reviewed and revised to take account of recent advances in *S. aureus* and enterotoxin research.

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